In Vitro and *In Vivo* Effect of ARQ 531 on Trk Family Kinases

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BACKGROUND

The tropomyosin receptor kinase (Trk) family contains TrkA, B and C that are encoded by the NTRK1, NTRK2, and NTRK3 genes, respectively. Trk family kinases have been shown to play a very important role in the development and physiological function of the nervous system. Chimeric Trk kinases resulting from gene fusions of NTRK genes are involved in the initiation and progression of various cancers including sarcoma, thyroid, salivary gland cancers, etc. Trk fusion kinases have been shown to be a driver in hematological malignancies, such as acute myeloid leukemia. Thus, targeting alterations of Trk family kinases offers a new path for therapeutic intervention. ARQ 531 was initially developed as a BTK inhibitor and is currently in a Phase I trial. With a distinct kinase profile, ARQ 531 also inhibits TrkA, B, and C.

MATERIALS AND METHODS

Cells and Cell Culture

KM12-luc cells (TPM3-NTRK1 fusion) were purchased from JCRB Cell Bank and K-562 cells (overexpressing TrkA) were purchased from ATCC. Cells were maintained at 37°C in a humidified atmosphere with 5% CO_2 according to manufacturer's recommendations.

Biochemical IC_{50} Determination

Biochemical IC_{50} values of ARQ 531 against TrkA, B and C were determined at an ATP concentration equal to its Km value.

Cell-based Kinase Assay

Inhibitory effect of ARQ 531 on Trk family kinases in cells were determined at DiscoverX using the PathHunter technology. U2OS cells are engineered to express TrkA, B, or C with a small peptide epitope (Prolink or PK) at the C-terminus. An Enzyme Acceptor (EA) tagged SH2 domain was co-expressed. Activation of the RTK-PK induces receptor dimerization, which leads to SH2-EA recruitment, and forces complementation of the two β-galactosidase enzyme fragments (EA and PK). The resulting functional enzyme hydrolyzes the substrate to generate a chemiluminescent signal. % Inhibition = 100% x (1 - (mean RLU of test sample - mean RLU of vehicle control) / (mean RLU of EC80 control - mean RLU of vehicle control)) and the IC_{50} was determined.

Cell Proliferation Assay

KM12-luc cells were seeded in 96-well tissue culture plates, incubated overnight and then treated with 3-fold serial dilutions of ARQ 531 at the starting concentration of 100 μM. Treated cells were incubated at 37°C for 72 hours in 5% CO_2. Thirty microliters of the mixture of MTS reagent (18.4 mg/ml) and PMS (0.92 mg/ml) at a ratio of 20:1 was added to each well, and the plates were incubated at 37°C for 4 hours in 5% CO_2. The absorbance was measured at 490 nM using a Victor Microplate reader. The IC_{50} was determined using Microsoft ExcelFit software in Activity Base.

The proliferation assay for Ba/F 3 cells expressing ETV6-NTRK1, -NTRK2, or –NTRK3 was performed using a CellTiter-Glo assay at Advanced Cellular Dynamic/Carnabiosciences.

Western Blot Analysis

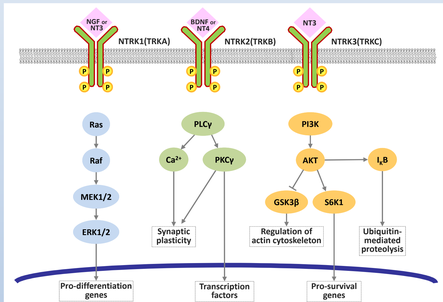
Cells were treated with different concentrations of ARQ 531 for various time periods. Proteins were extracted and resolved from extracts using SDS-PAGE followed by immunoblotting. Trk signaling pathway components were assessed. Images were captured using a FuJi LAS 3000.

In Vivo Efficacy Study

KM12-luc cells were inoculated subcutaneously into female BALB/c nude mice. When the average tumor volume reached 80-150 mm^3, 40 tumor-bearing mice were randomized into 4 groups: vehicle (n=10), ARQ 531 at 25 mg/kg (n=10), 50 mg/kg (n=10), and 75 mg/kg (n=10) for 2 weeks with a dosing schedule of 5 days on drug and 2 days off. ARQ 531 was formulated in Ethanol/Cremophor EL/Saline (1:1:8) and orally administered. The tumor length and width (mm) were measured using a digital caliper. Tumor volume (mm^3) [1/2 x (tumor length) x (tumor width)^2] and tumor growth inhibition (TGI) were determined. Results were expressed as mean ± SEM. Comparisons between the two groups were made by Dunnett's multi-comparison test, $P < 0.05$ was considered significant.

RESULTS

Trk Signaling Pathway



Khotskaya YB, et al. *Pharmacology & Therapeutics*. 2017;173:58-66.

Effect of ARQ 531 on TrkA, B and C

ARQ 531 potently inhibits Trk family kinases biochemically and cellularly

Assay	ARQ 531 IC_{50} (nM)		
	TrkA	TrkB	TrkC
Biochemical	1.3	1.8	1.8
Cellular (U2OS-Trks)	50.5	28.0	4.1

The Biochemical IC_{50} of ARQ 531 against TrkA, B, and C was determined at an ATP concentration equal to its Km. Cell-based kinase functional assay was performed using the PathHunter technology. The PathHunter U2OS cells expressing TrkA, TrkB or TrkC were treated with various concentrations of ARQ 531 and the IC_{50} values were determined.

ARQ 531 potently inhibited cell proliferation driven by Trk fusion kinases

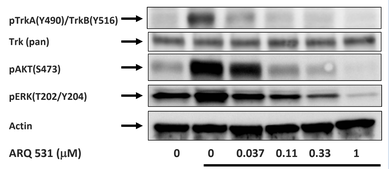
	Fusion Trk kinase	IC_{50} (μM)
KM-12(TPM3-NTRK1)	TPM3-TrkA	0.13
Ba/F3(ETV6-NTRK1)	TEL-TrkA	0.3
Ba/F3(ETV6-NTRK2)	TEL-TrkB	0.48
Ba/F3(ETV6-NTRK3)	TEL-TrkC	0.34

Anti-proliferation of ARQ 531 against Trk fusion driven KM12 colon cancer cells with TMP3-TrkA and Ba/F3 cells with ETV6-TrkA, -TrkB, and –TrkC was assessed. The IC_{50} values were determined. The IC_{50} for ARQ 531 against Ba/F(TEL) is 1.3 μM.

Effect of ARQ 531 on Trk Pathway

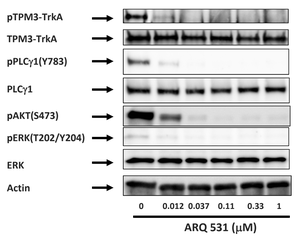
ARQ 531 potently inhibits TrkA and its downstream targets.

K-562 CML cells, overexpressing TrkA, were serum starved and treated with various concentrations of ARQ 531 for 2 hours, and then stimulated with NGF at 100 ng/mL for 10 minutes. pTrkA, pAKT and pERK, and total Trk were assessed using Western blot analysis.



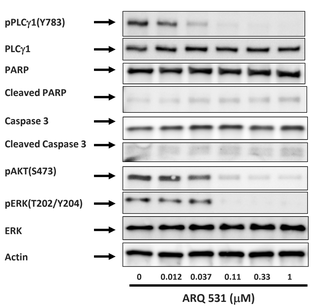
ARQ 531 markedly suppresses phosphorylation of TPM3-TrkA fusion kinase and its downstream signals

KM12-Luc colon cancer cells, TPM3-TrkA fusion, were serum starved and treated with various concentrations of ARQ 531 for 2 hours. TPM3-TrkA was immunoprecipitated and blotted with pTrkA or total TrkA antibodies. pPLCγ1, pAKT, and pERK were assessed using Western blot analysis.



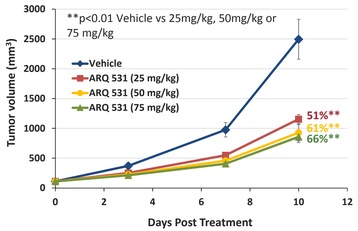
ARQ 531 markedly suppresses pPLCγ1, pAKT, and pERK and induces apoptotic response

KM12-Luc colon cancer cells, TPM3-TrkA fusion, were serum starved and treated with various concentrations of ARQ 531 for 24 hours. pPLCγ1, pAKT, pERK, Cleaved Caspase 3, and Cleaved PARP were assessed using Western blot analysis.

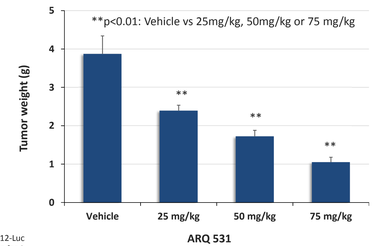


In Vivo Efficacy of ARQ 531 in Trk Fusion-driven KM12 Xenograft Model

ARQ 531 potently suppressed tumor growth with TGI of 51%, 61% and 66%, respectively

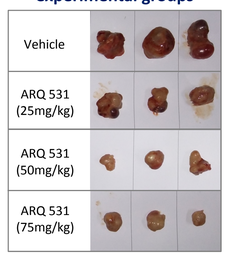


ARQ 531 significantly reduced tumor weight in comparison to vehicle treated group

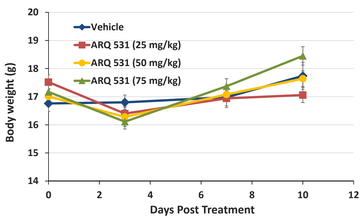


Anti-tumor activity was assessed by determining tumor volumes. Mice were inoculated with KM12-Luc cells (TMP3-TrkA) and orally administered ARQ 531 at 0 (Vehicle), 25 mg/kg, 50 mg/kg, or 75 mg/kg for 2 weeks at a schedule of 5 days on drug and 2 days off. There was no significant difference among treated groups.

Three representative tumors from each experimental groups



Mice in treated groups showed slight weight loss within in the first 3 days but recovered during treatment



CONCLUSIONS

- ARQ 531 potently inhibited Trk family kinases, as demonstrated in biochemical and cell-based assays
- ARQ 531 suppressed the Trk signal pathway in TrkA overexpressing K-562 cells and TPM3-TrkA fusion KM12 cells
- ARQ 531 potently inhibited proliferation of TPM3-TrkA fusion driven KM12 cells and Ba/F3 cells with ETV6-TrkA, -TrkB and -TrkC
- In a KM12 xenograft mouse model, ARQ 531 exhibited marked anti-tumor activity with a significant reduction of tumor volume and tumor weight
- The data provide a rationale for testing ARQ 531 in cancer patients with Trk fusion kinases